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(54) **Title:** AN IMPROVED METHOD FOR THE ENRICHMENT OF CIRCULATING TUMOR CELLS FROM DIAGNOSTIC LEUKAPHERESIS PRODUCTS

(57) **Abstract:** A method is provided for the analysis of DLA products incorporating aliquots of leukocytes for processing using CellSearch (DLA-CS). The Reduced Enrichment Reagent protocol (RER) is used to process $0.2x10^9$ leukocyte aliquots with 10-fold less enrichment reagents than DLA-CS. Using $1.0x10^9$ leukocyte aliquots a 4-fold increase in tumor cells compared to DLA-CS and a 24-fold increase compared to PB-CS was obtained. Using 10-fold less CellSearch capture reagent, we processed standard leukapheresis aliquots with no loss in tumor cell recovery, while attaining a higher purity. The method allows for 26% of the total leukapheresis sample to be processed using CellSearch reagents, enabling a sufficient number of CTC for tumor cell characterization in most metastatic prostate cancer patients. By the use of DNase the normally seen clumping of cells during the magnetic enrichment procedure is prevented, allowing better identification and further processing of the enriched CTC.

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An Improved Method for the Enrichment of Circulating Tumor Cells From Diagnostic Leukapheresis Products

BACKGROUND

5 Field of the Invention

The present invention relates to an improved method and process for rare cell enrichment and detection of circulating tumor cells (CTCs) from Diagnostic LeukApheresis (DLA).

Description of Related Art

10 The enumeration of circulating tumor cells (CTCs) from blood can be used for disease prognosis, treatment outcome, and disease relapse prediction. In most cases, immunomagnetic enrichment is employed to enrich the CTCs from hematopoietic cells. The most prominent example of enrichment methods is the FDA-cleared CellSearch system, which is designed to enrich CTCs from 7.5 mL blood samples. However, in many patients with 15 metastatic disease, the number of CTCs found in a standard 7.5 mL blood sample is too low for tumor cell characterization, while in patients with non-metastatic disease, the sensitivity and specificity are insufficient to determine the presence of disseminated cancer cells.

Several possibilities exist to increase the number of CTCs, such as capturing both the EpCAM positive as well as the EpCAM negative fraction of CTCs. However, the capture of 20 EpCAM-negative CTCs is hampered by a lack of markers, while also the prognostic value of these additional CTCs remains unclear. Another option is to draw blood closer to the tumor, for example from tumor-draining veins. Although more CTCs can be recovered this way, it is an invasive procedure normally only possible during surgery, and not necessarily relevant or feasible in the metastatic setting. The third option is to increase the evaluated blood volume, 25 for example by an in-vivo capture system or through Diagnostic LeukApheresis (DLA).

In a DLA procedure, continuous density separation is employed to harvest the mononuclear cells (MNCs), while most of the other blood components, such as plasma, erythrocytes, platelets, neutrophils, basophils, and eosinophils are returned to the patient. As CTCs have a similar density as MNCs, these are co-captured in the procedure. In a regular 30 leukapheresis procedure, more than five liters of blood are typically processed, whereas

Diagnostic Leukapheresis is a shortened procedure in which only two to five liters are processed, to minimize the burden on patients. The resulting samples can then be processed using the CellSearch system. Here aliquots of only $0.2x10^9$ white blood cells (WBC) are processed to ensure the sample can be analyzed. These $0.2x10^9$ aliquots only constitute 2-3% of the collected DLA sample.

The number of nucleated cells that are non-specifically enriched and end up in the analysis cartridge limits the amount of DLA that can be processed per sample. The processing of larger DLA aliquots frequently leads to excessively dense analysis cartridges, making identification of CTCs impossible. In this regard, it seems that the sample processing capability of the CellSearch system is underutilized due to the limitation in sample imaging and identification, something that could be addressed using a different imaging platform or by simply dividing the sample over several CellSearch cartridges. Another perspective is that the current methodology uses too much reagent per test.

As the CellSearch system was developed for the processing of 7.5 mL of whole blood, the initial enrichment takes place in a 10 mL volume. However, the volume taken up by cells in a DLA sample is much lower compared to blood, due to a large reduction of erythrocytes. With this in mind, the inventors of the present invention aimed to process DLA samples obtained from prostate cancer patients using standard CellSearch reagents, but with a reduced enrichment reagent protocol (RER). The inventors compared this approach to the processing of peripheral blood with CellSearch (PB-CS), the previously reported DLA with CellSearch (DLA-CS) and a reduced enrichment reagent protocol that enriches CTCs from $1.0x10^9$ WBC (RER+) instead of $0.2x10^9$ WBC.

BRIEF SUMMARY OF THE INVENTION

The standard CellSearch test was developed to detect CTCs in 7.5 ml of peripheral blood. In the majority of patients, the number of CTCs is insufficient for tumor characterization hence larger blood volumes are needed. DLA offers this opportunity and typically collects CTC from 1-5 liters of blood. The concentration of CTCs per tube of blood does not significantly decrease after DLA suggesting a fast replenishment from the metastatic sites and consequently processing multiple passages of the complete blood volume can lead to a further increase in the number of harvested CTCs. Adaptation of the CellSearch peripheral blood test

to DLA resulted in a DLA-CS protocol that uses DLA aliquots of $0.2x10^9$ leukocytes meaning that on average, the DLA product of 112 mL of blood could be processed per CellSearch test. Although one could perform greater than 30 DLA-CS tests to process the complete DLA product from five liters of blood, it would be cost and time prohibitive and makes subsequent interrogation of the tumor cells difficult. The major limitation to processing larger volumes was the large number of leukocytes remaining after enrichment.

Here, the inventors introduced the RER protocol, which is comparable to or better than the DLA-CS protocol, while using 10% of the enrichment reagents of a CellSearch test. The RER+ protocol uses 50% of the enrichment reagents to process a five times larger sample input, thereby overcoming the cost and time restrictions of the DLA-CS procedure.

The main difference between the DLA-CS and RER protocol lies in the reduced reagent volume, but there are some other differences as well. CellSearch is semi-automated and RER is a manual procedure, causing a higher operator dependence and likely more variability. Also, the magnetic incubation in the CellSearch system is eight times three minutes, and in RER three times 10 minutes magnetic incubation is used. In both the DLA-CS and RER protocols, staining is done using the same reagents at the same concentrations. However, RER uses a smaller staining volume. In the DLA-CS protocol, the system removes a portion of the unbound magnetic particles before the sample is transferred to the cartridge. The reason for this is that these hinder the imaging and identification of CTCs. In the RER protocol, this step is not necessary because there are much fewer ferrofluid particles present.

The enrichment of CTCs is followed by the identification of the captured cells and in many cases the isolation of single CTCs for further analysis. The RER protocol demonstrates the possibility of enriching CTCs from aliquots of $0.2x10^9$ WBC using only 10% of the CellSearch enrichment reagents while obtaining a comparable number of CTCs. We have also shown the possibility of processing aliquots of $1.0x10^9$ WBC while using 50% of the CellSearch enrichment reagents. As we have used less than 50% of the regular amount of reagents for the staining, with this approach a total sample of $2.0x10^9$ WBC could potentially be processed using a single CellSearch test. To analyze such a sample, a single CellSearch cartridge will not suffice. In most cases, either multiple cartridges will be needed, or sample imaging needs to be migrated to another system in which the cells can be distributed over a larger surface, such as an entire glass slide. For applications in which (initial) identification of all CTCs is not needed, such as

CTC culture or generation of patient-derived xenografts, this is not an issue. In these cases, only a portion of the resulting sample could be stained and imaged to estimate the total number of CTCs.

The lower number of total cells in the enriched samples when using the RER protocol is likely due to the lower amount of ferrofluid used. Surprisingly, there is no correlation seen in the total number of cells captured in DLA-CS and RER. A possible reason for this could be a threshold effect. By using a reduced reagent volume in samples where a high number of healthy cells are able to bind magnetic particles non-specifically, the reduced number of magnetic particles causes many of these cells to bind insufficient particles to be retained during separation. The specific antibody-antigen binding of the particles would in these cases outcompete the non-specific interaction, resulting in a retained binding efficiency for CTCs. If this is the case, an even lower reagent volume may further reduce non-specific capture without a loss of capture efficiency. As a too-high sample concentration or too-low magnetic particle concentration will inevitably lead to a reduction in CTC recovery, further optimization is needed.

To access the potential of diagnostic leukapheresis, the whole sample will need to be processed. We have shown that when using only 10% of the reagents normally used in a CellSearch test, it is possible to process a standard DLA aliquot of $0.2x10^9$ WBC sample with similar CTC recovery. At the same time, the amount of non-specifically enriched cells is significantly lowered, resulting in a better final sample purity. A proof of principle using six patient samples showed that this method also allows a five times larger DLA sample to be processed using only half of the standard CellSearch reagents, with only a slight reduction in recovery compared to a $0.2x10^9$ WBC sample. This workflow analysis opens up the possibility of processing even a much larger DLA sample in a cost-effective manner and is thereby the next step in realizing the full potential of diagnostic leukapheresis.

In one aspect, the invention provides a method for circulating tumor cell enrichment and enumeration workflow analysis in a subject, comprising: (a) obtaining (or providing) an apheresis sample containing CTCs (obtained) from a subject; (b) processing an apheresis aliquot in a system comprising: (i) concentrating the apheresis aliquot, preferably so as to reach a concentration of 0.05 $x10^9$-$0.5x10^9$, or 0.1 $x10^9$-$0.3x10^9$, more preferably $0.2x10^9$ white blood cells (WBC) per mL (e.g. by removal of plasma); (ii) incubating the (concentrated) aliquot

of (i) with immunomagnetic particles (comprising a CTC-antigen binding antibody); and (iii) separating the bound and unbound portion using a magnet; (c) optionally staining the sample (the bound portion obtained in step iii)) for cell identification, e.g. using fluorescent antibodies or fluorescent peptides; and (d) identification of CTC, e.g. using a fluorescence microscope
5 system, wherein the number of CTCs or their characteristics are used for disease prognosis, treatment outcome, and/or disease relapse prediction in the subject.

The method for circulating tumor cell enrichment and enumeration workflow analysis in a subject can also be termed as method for obtaining a composition enriched in circulating tumor cells (CTCs). Enrichment or enriched means that the obtained composition (bound
10 portion) has a higher concentration of CTCs relative to the starting sample/aliquot. The method may or may not comprise enumeration workflow analysis, which refers to enumerating the number of CTSs and/or their characteristics. Preferably, the method is ex vivo. Preferably, the concentrating in i. is performed by centrifuging. In a particularly preferred embodiment, step i) starts with pre-treating the apheresis aliquot (or sample) with
15 DNase. Concentration and incubation conditions will be clear to the skilled person. For example, DNase may be used in a concentration of 5-55, preferably 10-30 µg/ml, preferably in the presence of an activator such as MgSO4, preferably in a concentration of 5-50, preferably 10-30 µM (e.g. for a $2x10^9$ WBC aliquot of leukapheresis sample). Preferably, incubation time is 1-100, preferably 2-30, more preferably 5-15 min, preferably at 10-30
20 degrees Celsius, more preferably 15-25 degrees Celsius.

The fluorescent antibodies/peptides preferably bind CTC or CTC-antigen.

Instead of the apheresis aliquot, the method may also be applied on other types of samples, such as blood sample, cerebral spinal fluid sample, bone marrow fluid sample, ascites sample, urine sample, or pleural effusion sample.

25 In some embodiments the immunomagnetic particles are CellSearch Ferrofluid. The incubation with the immunomagnetic particles may be performed in the presence of a magnetic field. In some cases, controlled clustering of the immunomagnetic particles is performed during incubation.

The staining reagents may, for example, be CellSearch staining reagents. The imaging
30 microscope system may conveniently be a CellTracks Analyzer II. The obtained apheresis sample may be stored in CellSave vacutainers. The CellTracks Analyzer II system may include an analysis cartridge.

In some embodiments, the subject is a patient with metastatic hormone sensitive prostate cancer.

The apheresis may be Diagnostic LeukApheresis (DLA) and may, for example, be obtained in a Spectra Optia® system.

5 In some embodiments, the processed blood volume is 2 to 5 liters.

The DLA aliquot may, for example, consist of $0.2x10^9$ WBC or $1x10^9$ WBC or any value in the range $0.2x10^9$ WBC to $1x10^9$ WBC or even to $10x10^9$ WBC.

The solution used for fluorescent antibody staining may be a solution containing a permeabilization reagent, nuclear stain, staining reagent, and Cell Buffer for incubating the 10 sample at 37°C for 20 minutes.

The solution may be manually transferred to a CellSearch sample cartridge for placement into a CellSearch Magnest holder.

The CellSearch sample cartridge may be scanned in a CellTracks Analyzer II.

The CellTracks Analyzer II files may be processed using StarDist segmentation followed 15 by a deep learning approach.

In preferred embodiments, enumerating a sufficient number of CTCs provides a testing platform for an integrated use of clinical workflow.

In another aspect, the invention provides a diagnostic test kit for identification and/or characterization of one or more CTCs in an apheresis sample consisting of a workflow analysis 20 comprising: (a) optionally obtaining an aliquot of apheresis material; (b) a capture reagent; and (c) instructions for how to prepare the sample for analysis with a method as described above, where the kit characterizes CTCs used in disease prognosis, treatment outcome and disease relapse prediction in the subject.

The instructions may further include how to interrogate a sample, for example using a 25 CellTracks Analyzer II system.

In an alternative aspect, the invention provides a method for circulating tumor cell enrichment and enumeration workflow analysis in a subject, comprising: (a) obtaining (or providing) an apheresis sample containing CTCs (obtained) from a subject; (b) pretreating the sample using a nuclease, preferably DNase. This may reduce clumps and/or remove free DNA 30 in the sample; (c) enriching the tumor cells using immunomagnetic particles (comprising CTC-antigen binding antibody); (d) optionally staining the (enriched) sample for cell identification

using fluorescent antibodies/peptides; and (e) identification of CTC e.g. using a fluorescence microscope system, wherein the number of CTCs or their characteristics is used for disease prognosis, treatment outcome, and disease relapse prediction in the subject. In certain embodiments, this method may further comprise any of the optional method steps described above in relation to the first aspect of the invention.

The method for circulating tumor cell enrichment and enumeration workflow analysis in a subject can also be termed as method for obtaining a composition enriched in circulating tumor cells (CTCs). Enrichment or enriched means that the obtained composition (bound portion) has a higher concentration of CTCs relative to the starting sample/aliquot. The method may or may not comprise enumeration workflow analysis, which refers to enumerating the number of CTSs and/or their characteristics. Preferably, the method is ex vivo.

In a particularly preferred embodiment, step b) pretreats the sample/aliquot with DNase. Concentration and incubation conditions will be clear to the skilled person. For example, DNase may be used in a concentration of 5-55, preferably 10-30 µg/ml, preferably in the presence of $MgSO_4$ preferably in a concentration of 5-50, preferably 10-30 µg/ml (e.g. for a $2x10^9$ WBC aliquot of leukapheresis sample). Preferably, incubation time is 1-100, preferably 2-30, more preferably 5-15 min, preferably at 10-30 degrees Celsius, more preferably 15-25 degrees Celsius.

Preferably, step b. further includes concentrating the apheresis aliquot/sample so as to reach a concentration of $0.05\ x10^9$-$0.5x10^9$, or $0.1\ x10^9$-$0.3x10^9$, more preferably $0.2x10^9$ white blood cells (WBC) per mL (e.g. by removal of plasma). Preferably, the concentrating is performed by centrifuging.

The fluorescent antibodies/peptides preferably bind CTC or CTC-antigen.

Instead of the apheresis aliquot, the method may also be applied on other types of samples, such as blood sample, cerebral spinal fluid sample, bone marrow fluid sample, ascites sample, urine sample, or pleural effusion sample.

In a further alternative aspect, the invention provides a diagnostic test kit for identification and/or characterization of one or more CTCs in an apheresis sample consisting of a workflow analysis comprising: (a) an aliquot of apheresis material; (b) a capture reagent; and (c) instructions for how to prepare the sample for analysis with a method as described in

the preceding paragraph, where the kit characterizes CTCs used in disease prognosis, treatment outcome and disease relapse prediction in the subject.

BRIEF DESCRIPTION OF THE FIGURES

Some embodiments of the present invention are illustrated as an example and are not limited by the figures of the accompanying drawings:

Fig 1 A schematic representation of the RER procedure to process DLA aliquots. The bottom row indicates the time for each step. The red/blue rectangle represents the BD iMag (cell separation magnet). FF: ferrofluid, CE: capture enhancement reagent.

Fig 2 Panel A: Average number of cells and platelets per mL and **Panel B:** Percentage of the volume consisting of cells and platelets in blood and DLA as collected as well as during magnetic particle incubation in PB-CS, DLA-CS, and RER procedures.

Fig 3 (A) Comparison of CTC recovery between DLA-CS and RER procedures using $0.2x10^9$ WBC from 30 DLA samples of metastatic prostate cancer patients. **(B)** Number of CTC detected in 30 peripheral blood and regular DLA samples using CellSearch, RER, and RER+ (6 samples) procedures.

Fig 4 (A) Comparison of the total number of cells after processing of DLA samples with CS-DLA and RER protocols for 30 leukapheresis samples of prostate cancer patients. **(B)** The total number of cells after processing whole blood and DLA samples using CellSearch, RER, and RER+ (6 samples) protocols.

Fig 5 (A) Final sample purity and **(B)** relative CTC recovery compared to blood. Results represent final samples as found after enrichment of 7.5 mL of blood, $0.2x10^9$ or $1.0x10^9$ WBC from DLA and processed using CellSearch, RER, or RER+.

Fig 6 Snippets of the DAPI channel showing nucleated cells in the enriched samples containing 100,000, 200,000, 300,000, and 400,000 cells.

Fig 7 (A) Coupled number of CTC and the total number of cells with both methods for all 30 samples and **(B)** relative number of CTC and the total number of cells for all 30 samples.

Fig 8 Graph showing recovery of CTC from 19 DLA samples of prostate cancer patients with or without DNase pre-treatment.

Fig 9 Graph showing a comparison of the total number of cells after processing of 19 DLA samples with or without DNase pre-treatment.

Fig 10 Representation of images having the nuclear stain DAPI from samples after enrichment with and without DNase pre-treatment. Rows contain images from the same patient sample.

DETAILED DESCRIPTION OF INVENTION

5 The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the term “and/or” includes any and all combinations of one or more of the associated listed items. As used herein, the singular forms “a,” “an,” and “the” are intended to include the plural forms as well as the singular forms, unless the context clearly indicates otherwise. It will be further understood 10 that the terms “compromises” and/or “compromising,” when used in this specification, specify the presence of stated features, steps, operations, elements, components, and/or groups thereof, but do not preclude the presence or addition of one or more other features, steps, operations, elements, components, and/or groups thereof.

Unless otherwise defined, all terms (including technical and scientific terms) used herein 15 have the same meaning as commonly understood by one having ordinary skill in the art to which this invention belongs. It will be further understood that terms, such as those used in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the relevant art and the present disclosure and will not be interpreted in an idealized or overly formal sense unless expressly so defined herein.

20 In describing the invention, it will also be understood that a number of techniques and steps are disclosed. Each of these has individual benefit and each can also be used in conjunction with one or more, or in some cases all, of the other disclosed techniques. Accordingly, for the sake of clarity, this description will refrain from repeating every possible combination of the individual steps in an unnecessary fashion. Nevertheless, the specification 25 and claims should be read with the understanding that such combinations are entirely within the scope of the invention and claims.

The median number of circulating tumor cells (CTCs) detected in 7.5 mL of peripheral blood by CellSearch (PB-CS) in patients with metastatic prostate cancer is in the order of 1-10, which means many samples have insufficient tumor cells for comprehensive characterization. 30 A significant increase in blood volume is obtained through Diagnostic LeukApheresis (DLA),

however, only 2-3% of the DLA product can be processed per CellSearch test, limiting the gain in blood volume.

Patient samples

DLA samples were obtained from 28 metastatic Hormone Sensitive Prostate Cancer patients (mHSPC) before initiation of treatment and with greater than 2 CTCs in a 7.5 mL sample of blood. In two cases, patients underwent a second leukapheresis procedure after becoming castration-resistant resulting in a total of 30 samples. Leukapheresis was performed per the optimized procedure described by Mout et al. on a Spectra Optia (Terumo, Lakewood, Co, USA). Samples were collected in accordance with the Declaration of Helsinki as part of a study approved by the medical ethical committee of the Erasmus Medical Center.

Relative recovery, cell concentration, and cell volume calculation

To compare the CTC recovery from DLA to that found in PB-CS (CTC_{PB}), the expected number of CTC (CTC_{exp}) was calculated using equation 1

$$CTC_{exp} = \frac{MNC_{DLA}}{MNC_{PB}} * CTC_{PB} \quad (1)$$

where MNC_{DLA} and MNC_{PB} are the total number of MNCs in the processed DLA aliquot and CS-PB sample respectively.

During a DLA procedure, the MNC population is targeted for extraction. The sample however contains impurities; some erythrocytes, granulocytes, and platelets are co-captured during the procedure. To compare the cell density in RER to that of CS-PB and CS-DLA, we calculated the total number of cells per mL as well as what percentage of the volume is taken up by cells (volume fraction) at the time of magnetic particle incubation. For this calculation we have represented the different cell types as having a volume of: erythrocytes 90 pL, lymphocytes 187 pL, monocytes 413 pL, neutrophils 299 pL, eosinophils 344 pL, basophils 344 pL, and platelets 10.5 pL. In these calculations we accounted for the following volume changes: Before processing on the CellSearch AutoPrep, a 7.5 mL whole blood or a DLA sample is diluted to 14 mL and centrifuged. The AutoPrep then aspirates the diluted plasma and some of the platelets, leaving about 4 mL. This sample is diluted again with 6 mL of CellSearch dilution buffer before the magnetic particles are added. In RER processing, the DLA samples are concentrated by the removal of plasma to reach a concentration of 0.2×10^9 WBC/mL.

Differential blood counts of DLA and whole blood samples were taken on the same day and determined on a DxH 500 hematology analyzer (Beckman Coulter, Utrecht, The Netherlands). The number of blood cells as well as their total volume per mL during magnetic particle incubation was then calculated for the blood and DLA product during PB-CS, DLA-CS, and RER processing.

CellSearch processing

Patient blood samples were stored in CellSave vacutainers (Menarini-Silicon Biosystems, Bologna, Italy) and processed using the CellSearch system according to the manufacturer's instructions using the CTC-kit (Menarini-Silicon Biosystems, Bologna, Italy). DLA samples were stored in CellSave vacutainers in 10 mL aliquots and shipped overnight to the University of Twente. For DLA samples, an aliquot containing $0.2x10^9$ WBC was placed in a 15mL conical tube (Menarini-Silicon Biosystems, Bologna, Italy) and processed on the CellSearch Autoprep according to the manufacturer's instructions whenever possible using the CTC-kit. All samples were processed within 48 hours after collection.

Reduced enrichment reagent procedures

Enrichment: For the RER procedure, an aliquot containing $0.2x10^9$ WBC was placed into a 12x75mm 4.5 mL centrifuge tube (Greiner bio, Alphen aan de Rijn, The Netherlands) and centrifuged at 400*g* for 5 minutes. Subsequently, the supernatant was aspirated until 1 mL remained. The sample was then incubated with 15 µL CellSearch ferrofluid for 10 minutes in a magnet (iMag, Becton Dickinson, San Jose, CA, USA) after which 15µL CellSearch capture enhancement reagent was added. The sample was mixed and incubated twice more for ten minutes in the magnet, mixing again after each incubation. Next, the sample was supplemented with 2ml 'Cell buffer' (phosphate buffered saline (Merck, Darmstadt, Germany) supplemented with bovine serum albumin (Merck), EDTA (Merck), casein (Merck) and mouse serum (Invitrogen, Carlsbad, USA) and placed in the magnet for 20 minutes after which the unbound fraction was aspirated using a glass Pasteur pipet and syringe pump set to 1 mL/min. The bound fraction was resuspended in 1mL of cell buffer before performing a second separation of 10 minutes in the magnet.

For the RER+ procedure, samples containing $1.0x10^9$ WBC were first incubated with 20 µg/mL DNase I (Roche, Basel, Switzerland) together with 20 µM $MgSO_4$ to prevent aggregation. The enrichment was performed analogously to the RER procedure. However, as

the RER+ protocol uses a five times larger sample input, all volumes up to the separation step in figure 1 were multiplied by 5, and larger consumables were used where needed. Specifically, aspirating supernatant to 5 mL in a 12 mL round bottom tube (Greiner bio), and using 75 µL CellSearch ferrofluid and 75 µL CellSearch capture enhancement. RER+ samples were transferred to 12x75mm centrifuge tubes after the initial separation and processed further using the standard RER protocol. Staining of RER and RER+ samples was performed identically.

DNase as an effective prevention of leukocyte aggregates in the immunomagnetic processing of leukapheresis material: Next to the sheer number of WBC that is non-specially co-captured in the immune-magnetic enrichment of CTC, also the presence of cell aggregates limits the tolerable cell density during CTC identification, and with that the processable DLA volume. Additionally, these aggregates prevent efficient single-cell analysis and the identification of pre-existing tumor-associated clusters. Close examination of these aggregates has revealed that the reason for their aggregation is the presence of free DNA in the resulting samples.

Here, the inventors of the present invention introduced the application of a DNase pre-treatment for DLA material prior to sample processing. With this pre-treatment, the free DNA material in the DLA material is removed, while the DNA material in the CTC and WBC is preserved. The improved sample quality of the resulting samples will allow the processing of more samples as automated analysis can be better applied to the clearly distinguishable cells in the DNase pre-treated samples.

Proof of principle results: To first examine if the DNase pre-treatment has an effect on the CTC capture we compared the number of CTC found per $0.2x10^9$ WBC aliquots of DLA material from 18 metastatic prostate cancer patients. For this, we added DNase at a final concentration of 20µg/ml as well as $MgSO_4$ at a final concentration of 20µM to an $0.2x10^9$ WBC aliquot of CellSave fixated leukapheresis sample. After 10min of incubation at room temperature, the magnetic enrichment procedure was performed for the sample pre-treated with DNase as well as an identical sample from the same patient not pre-treated with DNase. As shown in Fig 8, the results indicate no effect of DNase on the number of CTCs captured. Paired t-test on log transformed data did not show a significant difference in CTC recovery (p=0.127) while linear regression on log-transformed data resulted in a regression of $\log_{10}$

(CTC_{DNase}) = -0.288+1.114 $\log_{10}$ ($CTC_{No\text{-}DNase}$) and a high correlation with an R^2 of 0.982, indicating the procedure with DNase pre-treatment has similar CTC recovery as the procedure without DNase pre-treatment.

The issue with the immunomagnetic enrichment of CTCs from DLA material lies in the non-specific enrichment of healthy cells. To examine the effect of DNase on the co-enrichment of healthy cells we evaluated the total number of cells present in the enriched samples with and without the DNase pre-treatment. The total number of co-enriched WBCs with and without DNase pretreatment is shown in Fig 9. Paired t-test on log-transformed data showed a slightly significant reduction in the number of WBCs co-enriched when using the DNase pretreatment (p=0.0492). Linear regression on log-transformed data showed that the number of cells captured with and without DNase pre-treatment is slightly correlated (R^2=0.248), with a linear regression of $\log_{10}(WBC_{DNase}) = 0.826 + 0.791 \log_{10}(WBC_{No\text{-}DNase})$.

The ease of identification of CTC in these enriched samples is however not only dependent on the number of cells, but also on the ability to assess the identity of these cells individually. In Fig 10, a gallery of single images from 4 different prostate cancer DLA samples enriched with or without DNase pre-treatment is shown, demonstrating the improved sample quality and absence of cell aggregates due to the application of DNase in this setting.

Staining: After aspiration of the unbound fraction the sample was resuspended in 300 µL staining solution, consisting of 50 µL permeabilization reagent, 50 µL nuclear stain, and 50 µL staining reagent, supplemented with 150 µL Cell buffer. All reagents were taken from a CTC kit (MSB). The sample was incubated at 37°C for 20 minutes, after which 700 µL Cell buffer was added and the sample was placed in the magnet for 10 minutes. The unbound fraction was aspirated and the bound fraction was resuspended in 150 µL CellSearch fixation reagent with 175 µL phosphate-buffered saline. The sample was then manually transferred to a CellSearch sample cartridge using a 230 mm glass Pasteur pipet pre-coated with bovine serum albumin and placed into a CellSearch Magnest.

A schematic representation of the 0.2×10^9 WBC RER procedure steps and times is shown in figure 1.

<u>Enumeration of CTC and total cell number</u>

PB-CS, DLA-CS, RER, and RER+ samples were scanned using the CellTracks Analyzer II. To minimize inter-reviewer variability the number of CTC in each pair of samples CTCs were scored by the same reviewer using the standard CellTracks Analyzer II software. The total number of cells in each cartridge was determined by processing the CellTracks Analyzer 5 archives using StarDist segmentation followed by a deep learning approach.

Statistics

To make the linear regression robust against the large spread in the number of CTCs as well as the total number of cells, both counts were log-transformed before regression. Because CTC counts are not normally distributed, we used the non-parametric Wilcoxon 10 Signed Ranks Test to compare the number of CTCs as well as the total number of cells enriched using each method. All tests were performed using Origin 2019b (OriginLab Corporation, Northampton, MA, USA).

Cell volume in blood and DLA product

15 To assess whether increasing the concentration of WBC in the DLA product before enrichment results in a more concentrated sample compared to peripheral blood, we calculated the number and volume composition for both sample types based on differential blood counts. Results are shown in figure 2.

The number of cells and platelets per mL during magnetic particle incubation in CS-DLA 20 is on average only 7% (median 7%, range 4% to 9%) of that in PB-CS. In the RER protocol, the number of cells and platelets per mL is about equal to that in PB-CS (average 98%, median 96%, range 59% to 131%).

The percentage of volume taken up by cells and platelets (volume fraction) in PB-CS was on average 26% (median 26%, range 20% to 32%), and for the DLA-CS procedure on 25 average 1.5% (median 1.4%, range 1.1% to 2.2%). In the RER procedure, the volume fraction is 16% (median 14%, range 11% to 23%), which is much closer to, but still below the volume fraction in PB-CS.

The number of cells and platelets per mL in the RER procedure is similar to that in CS-PB, while the cell volume concentration is below that of PB-CS. As the concentration of capture reagents 30 in the assay is the same for all three procedures, the capture reagent per cell is approximately the same for PB-CS and RER, and approximately 12-fold higher in PB-CS. The capture reagent

per cell volume is compared to PB-CS approximately 2-fold higher in the RER protocol, and approximately 10-fold higher in DLA-CS.

CTC recovery

Aliquots of 30 DLA samples were processed using DLA-CS, and RER protocols, together with peripheral blood samples using the PB-CS protocol. In figure 3A the number of recovered CTCs after DLA-CS and RER processing are compared. Linear regression was performed on log-transformed data to be robust against outliers. This resulted in a regression of $\log_{10}(CTC_{RER}) = -0.036+0.977 \log_{10}(CTC_{DLA\text{-}CS})$ with an R^2 of 0.95. Using the non-parametric Wilcoxon Signed Ranks Test the paired samples did not show a significant difference (p=0.957), indicating the RER procedure has similar CTC recovery as the DLA-CS procedure. To make a first step toward processing the entire DLA sample, we evaluated the use of RER adjusted for a 5-fold larger sample size (RER+) using six out of the thirty patient samples. On average, this 5-fold larger sample size accounted for 13% of the available sample.

The DLA-CS of an 0.2×10^9 WBC aliquot leads to a median 5.6-fold (mean 5.6, range 1.4 to 14.6-fold) increase in CTC compared to the 7.5 mL whole blood PB-CS. RER leads to a similar increase (median 4.4, mean 5.5, range 2 to 18.6-fold). In the six samples for which also the RER+ protocol was performed, another 3.8-fold median increase in CTC was observed compared to DLA-CS and 3.4-fold compared to RER, or a total median increase of 19.3-fold (mean 22.9, range 4.3 - 50.1 to fold) in CTC compared to PB-CS. The number of CTC found in all samples with the different enrichment methods is shown in figure 3B.

White blood cell carry-over

We expected the lower amount of reagents to result in a lower non-specific binding of magnetic particles, and therefore a lower non-specific cell capture. To examine this, we evaluated the total number of cells present in the enriched samples, as shown in figure 4. Paired t-test shows a statistically significant difference in the two populations ($p<0.001$) with a lower number of cells after RER processing (mean 59,590, median 44,388) than after DLA-CS processing (mean 152,624, median 134,185). Surprisingly, as shown in figure 4A, we find that the number of cells for one of the methods is not predictive of the number of cells with the other method, as there is no correlation (R^2=0.003) in the number of cells between the two methods, with a linear regression of $\log_{10}(cells_{RER}) = 4.336 + 0.056 \log_{10}(cells_{DLA\text{-}CS})$.

The total number of cells as shown in figure 4B showed a median 26.1-fold (mean 51.6, range 0.03 – 288.4-fold) increase when moving from PB-CS to DLA-CS. Comparing DLA-CS to RER a median reduction in total cells of 2.7-fold was observed (mean 6.6, range 0.2 – 62.7-fold, Wilcoxon Signed Ranks test, p<0.001). Comparing RER to RER+ for the six samples where both were performed, a median increase of 5.2-fold was observed (mean 8.6, range 0.75 - 30.3-fold), compared to a 5-fold larger number of cells processed. Most of the RER+ samples contain too many cells to be imaged when placed into a single CellSearch cartridge, and two to three cartridges per sample were used. A gallery of different cell densities is shown in supplementary figure 6. We observed no correlation between the change in CTCs and total cell numbers, see supplementary figure 7.

Purity and relative recovery

As the number of CTCs remained similar while the total number of cells in the final sample decreased, the CTCs to total cell fraction of RER samples was improved compared to DLA-CS samples. In figure 5A the sample purity of all matched blood and DLA samples processed with CellSearch, RER, and RER+ are shown. It can be seen that although the number of CTCs increased, the purity of the resulting samples when processing DLA using CellSearch was reduced compared to blood (Wilcoxon Signed Ranks test, p<0.001). However, when using RER a median 2.2-fold improvement (mean 5.8, range 0.3 fold 41.2-fold, Wilcoxon Signed Ranks test, p=0.004) in sample purity was found.

In figure 5B, it can be seen that both the DLA-CS and RER resulted in a relative recovery of approximately 50% (mean 53 and 54%, median 46 and 43%) compared to PB-CS, which indicates a loss of CTC in the DLA procedure. The increase in volume to $1.0x10^9$ in RER+ seems to show a small reduction in relative recovery (mean 39%, median 44%), which might also be a result of the small sample size.

Discussion

The standard CellSearch test was developed to detect CTCs in 7.5 ml of peripheral blood. In the majority of patients, the number of CTCs is insufficient for tumor characterization hence larger blood volumes are needed[6]. DLA offers this opportunity and typically collects CTC from 1-5 liters of blood. The concentration of CTCs per tube of blood does not significantly decrease after DLA suggesting a fast replenishment from the metastatic sites and consequently processing multiple passages of the complete blood volume can lead to a further

increase in the number of harvested CTCs. Adaptation of the CellSearch peripheral blood test to DLA resulted in a DLA-CS protocol that uses DLA aliquots of $0.2x10^9$ leukocytes meaning that on average, the DLA product of 112 mL of blood could be processed per CellSearch test. Although one could perform greater than 30 DLA-CS tests to process the complete DLA product from five liters of blood, it would be cost and time prohibitive and makes subsequent interrogation of the tumor cells difficult. The major limitation to processing larger volumes was the large number of leukocytes remaining after enrichment.

Here, we introduced the RER protocol, which is comparable to or better than the DLA-CS protocol, while using 10% of the enrichment reagents of a CellSearch test. The RER+ protocol uses 50% of the enrichment reagents to process a five times larger sample input, thereby overcoming the cost and time restrictions of the DLA-CS procedure.

The main difference between the DLA-CS and RER protocol lies in the reduced reagent volume, but there are some other differences as well. CellSearch is semi-automated and RER is a manual procedure, causing a higher operator dependence and likely more variability. Also, the magnetic incubation in the CellSearch system is eight times three minutes, and in RER three times 10 minutes magnetic incubation is used. In both the DLA-CS and RER protocols, staining is done using the same reagents at the same concentrations. However, RER uses a smaller staining volume and staining is performed at 37°C. In the DLA-CS protocol, the system removes a portion of the unbound magnetic particles before the sample is transferred to the cartridge. The reason for this is that these hinder the imaging and identification of CTCs. In the RER protocol, this step is not necessary because there are much fewer ferrofluid particles present.

The enrichment of CTCs is followed by the identification of the captured cells and in many cases the isolation of single CTCs for further analysis. The RER protocol demonstrates the possibility of enriching CTCs from aliquots of $0.2x10^9$ WBC using only 10% of the CellSearch enrichment reagents while obtaining a comparable number of CTCs. We have also shown the possibility of processing aliquots of $1.0x10^9$ WBC while using 50% of the CellSearch enrichment reagents. As we have used less than 50% of the regular amount of reagents for the staining, with this approach a total sample of $2.0x10^9$ WBC could potentially be processed using a single CellSearch test. To analyze such a sample, a single CellSearch cartridge will not suffice. In most cases, either multiple cartridges will be needed, or sample imaging needs to be migrated to

another system in which the cells can be distributed over a larger surface, such as an entire glass slide. For applications in which (initial) identification of all CTCs is not needed, such as CTC culture or generation of patient-derived xenografts, this is not an issue. In these cases, only a portion of the resulting sample could be stained and imaged to estimate the total number of CTCs.

The lower number of total cells in the enriched samples when using the RER protocol is likely due to the lower amount of ferrofluid used. Surprisingly, there is no correlation seen in the total number of cells captured in DLA-CS and RER. A possible reason for this could be a threshold effect: By using a reduced reagent volume, in samples where a high number of healthy cells is able to bind magnetic particles non-specifically, the reduced number of magnetic particles causes many of these cells to bind insufficient particles to be retained during separation. The specific antibody-antigen binding of the particles would in these cases outcompete the non-specific interaction, resulting in a retained binding efficiency for CTCs. If this is the case, an even lower reagent volume may further reduce non-specific capture without a loss of capture efficiency. As a too-high sample concentration or too-low magnetic particle concentration will inevitably lead to a reduction in CTC recovery, further optimization is needed.

To access the potential of diagnostic leukapheresis, the whole sample will need to be processed. We have shown that when using only 10% of the reagents normally used in a CellSearch test, it is possible to process a standard DLA aliquot of $0.2x10^9$ WBC sample with similar CTC recovery. At the same time, the amount of non-specifically enriched cells is significantly lowered, resulting in a better final sample purity. A proof of principle using six patient samples showed that this method also allows a five times larger DLA sample to be processed using only half of the standard CellSearch reagents, with only a slight reduction in recovery compared to a $0.2x10^9$ WBC sample. This work opens up the possibility of processing even a much larger DLA sample in a cost-effective manner and is thereby the next step in realizing the full potential of diagnostic leukapheresis.

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CLAIMS

1. A method for circulating tumor cell enrichment and enumeration workflow analysis comprising:
    a. providing an apheresis sample containing Circulating Tumor Cells (CTCs) obtained from a subject;
    b. processing an apheresis aliquot in a system comprising:
        i. concentrating the apheresis aliquot by removal of plasma so as to reach a concentration of 0.05 $x10^9$-$0.5x10^9$, more preferably $0.2x10^9$ white blood cells (WBC) per mL;
        ii. incubating the concentrated apheresis aliquot of (i) with immunomagnetic particles that comprise a CTC-antigen binding antibody; and
        iii. separating the bound and unbound portion using a magnet;
    c. staining the bound portion for cell identification using fluorescent antibodies; and
    d. identification of CTC using a fluorescence microscope system,

    wherein the number of CTCs or their characteristics are used for disease prognosis, treatment outcome, and/or disease relapse prediction in the subject.
2. The method of claim 1 where the used immunomagnetic particles are ferrofluid particles.
3. The method of claim 1 or claim 2, where the incubation with the immunomagnetic particles is performed in the presence of a magnetic field.
4. The method of any of claims 1 to 3, where controlled clustering of the immunomagnetic particles is performed during incubation.
5. The method of any preceding claim, where the solution used for fluorescent antibody staining contains a permeabilization reagent, nuclear stain, and fluorescent staining reagents.
6. The method of any preceding claim, where the fluorescence microscope system is an imaging microscope system.
7. The method of any preceding claim, where the obtained apheresis sample is stored.
8. The method of any preceding claim, where the subject is a patient with metastatic hormone sensitive prostate cancer.

9. The method of any preceding claim, where the apheresis is Diagnostic LeukApheresis (DLA).
10. The method of claim 9 where the processed blood volume is 2 to 5 liters.
11. The method of claim 9 or claim 10, where a DLA aliquot consists of from $0.2x10^9$
5 white blood cells (WBC) to $10x10^9$ WBC.
12. The method of any one of claims 6-11, where the imaging microscope system files are processed using segmentation followed by a deep learning approach for integrated use in clinical workflow.
13. A method for circulating tumor cell enrichment and enumeration workflow analysis
10 comprising:
    a. providing an apheresis sample containing CTCs obtained from a subject;
    b. pretreating the sample using a DNase;
    c. enriching the tumor cells using immunomagnetic particles that comprise CTC-antigen binding antibody;
15 d. staining the (enriched) sample for cell identification using fluorescent antibodies; and
    e. identification of CTC using a fluorescence microscope system,

    wherein the number of CTCs or their characteristics is used for disease prognosis, treatment outcome, and disease relapse prediction in the subject.
20 14. The method of claim 13, further comprising the step(s) defined in any of claims 2 to 12.

Fig 1

Make 1ml	Add FF	Incubate	Add CE	Incubate	Separate	Wash	Stain	Wash	Transfer
10 min	1 min	10 min	1 min	2x10 min	20 min	10 min	20 min	10 min	1 min

Fig 2

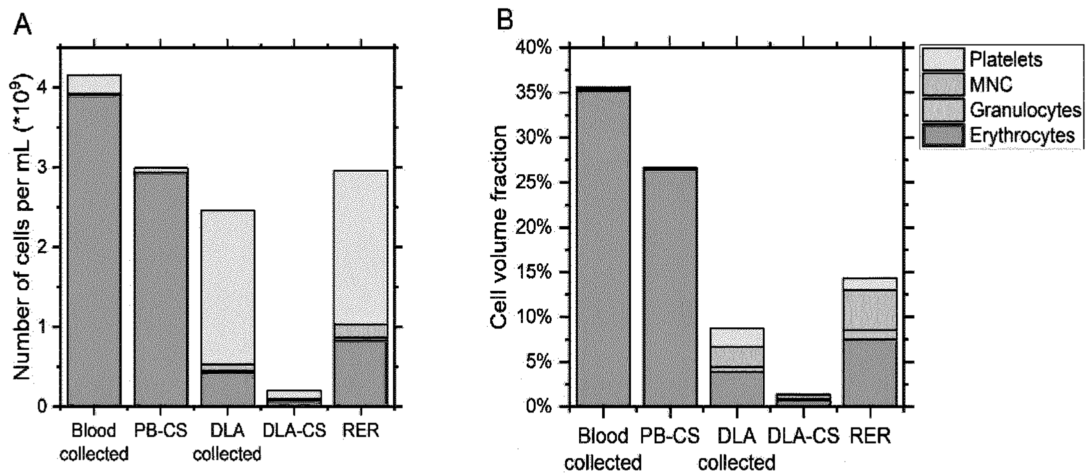


Fig 3

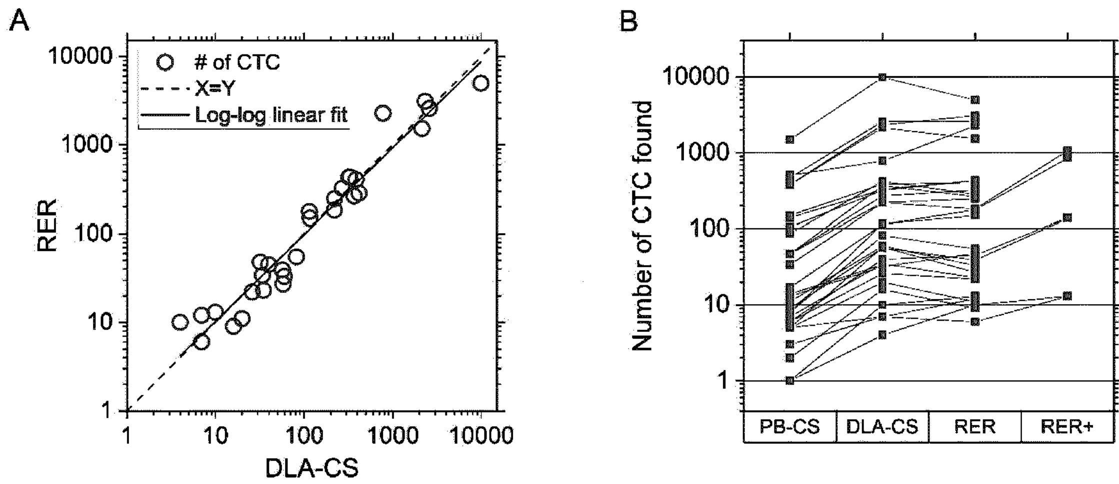


Fig 4

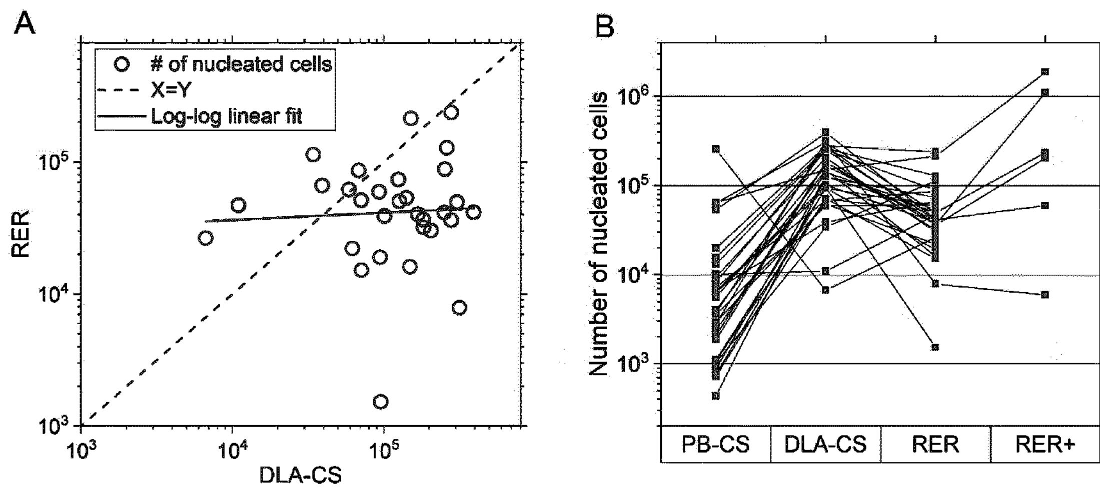


Fig 5

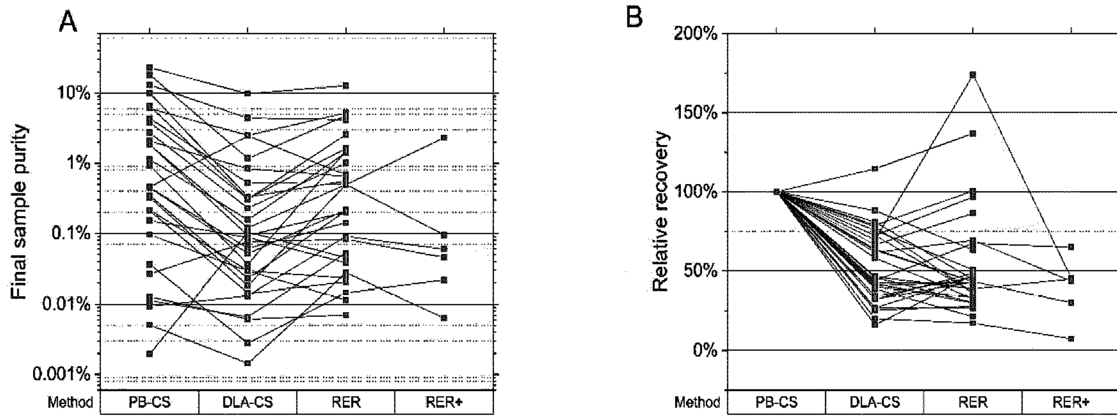
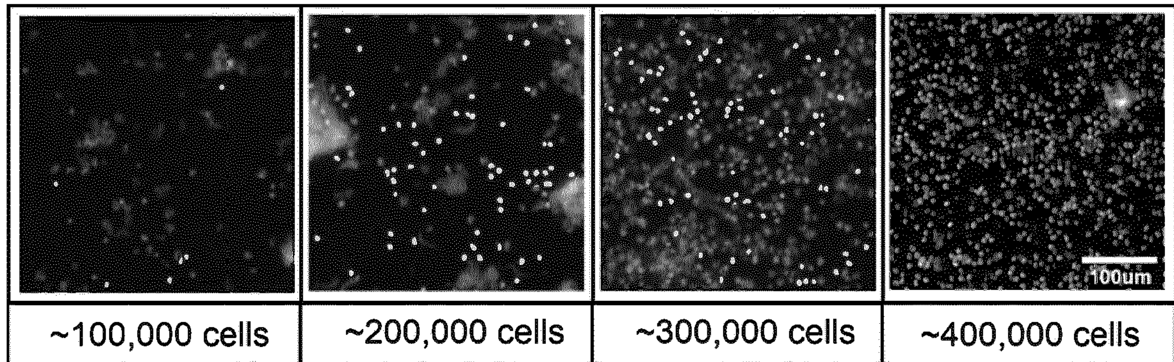


Fig 6



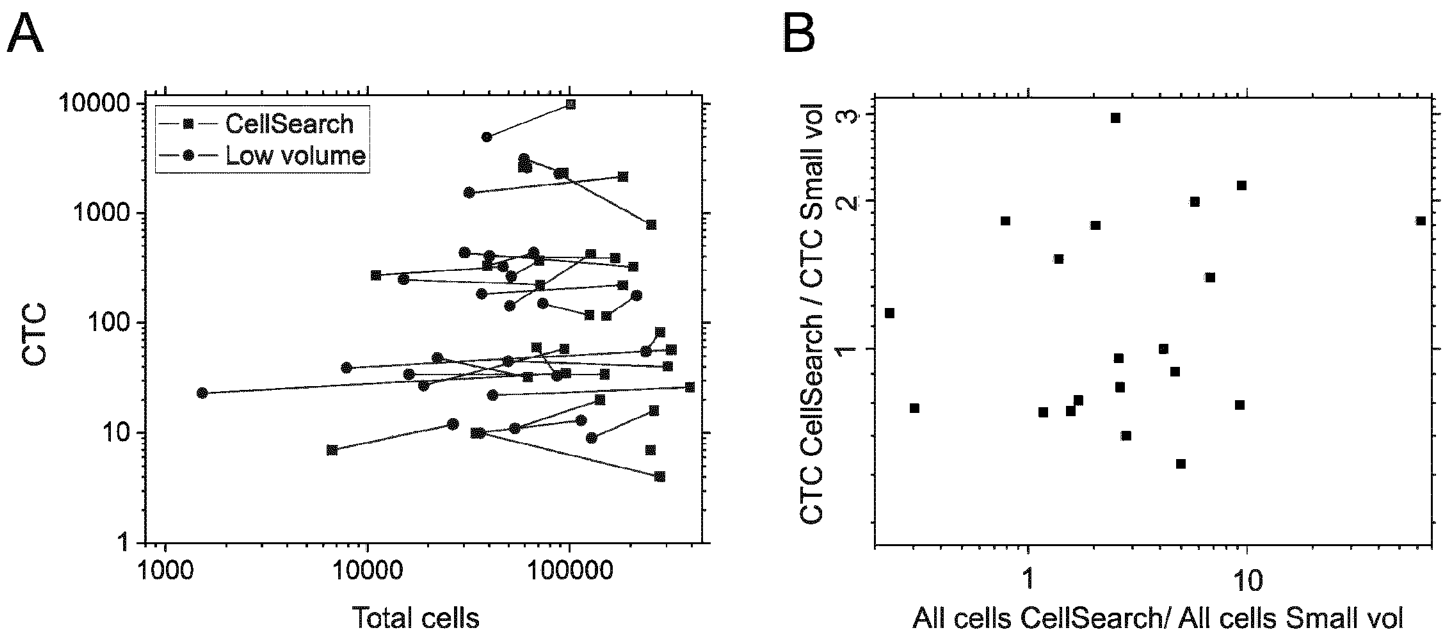


Fig. 7

Fig 8

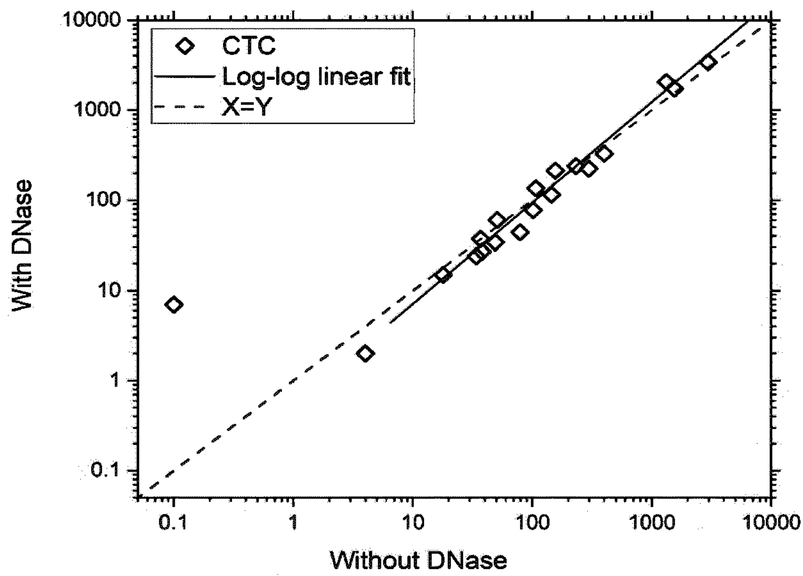


Fig 9

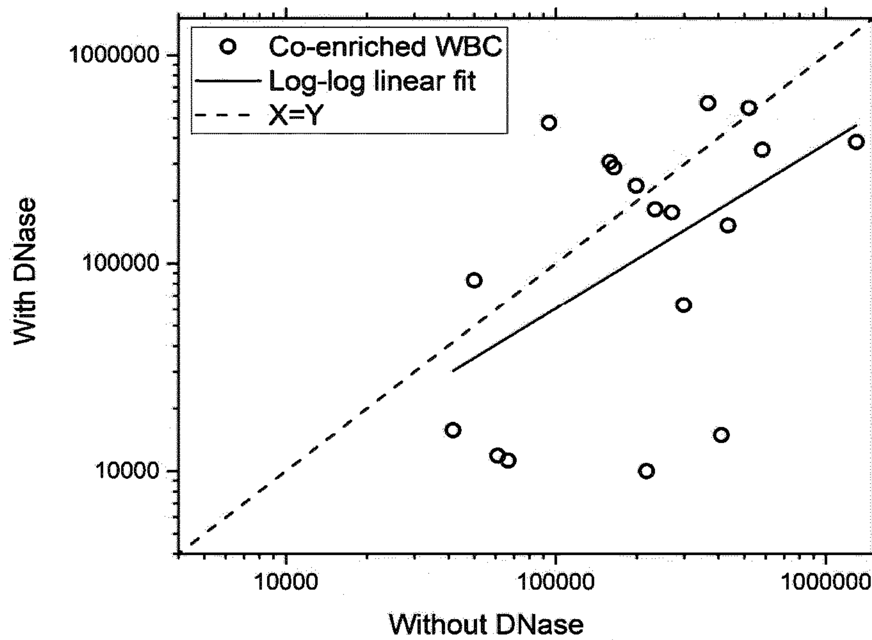
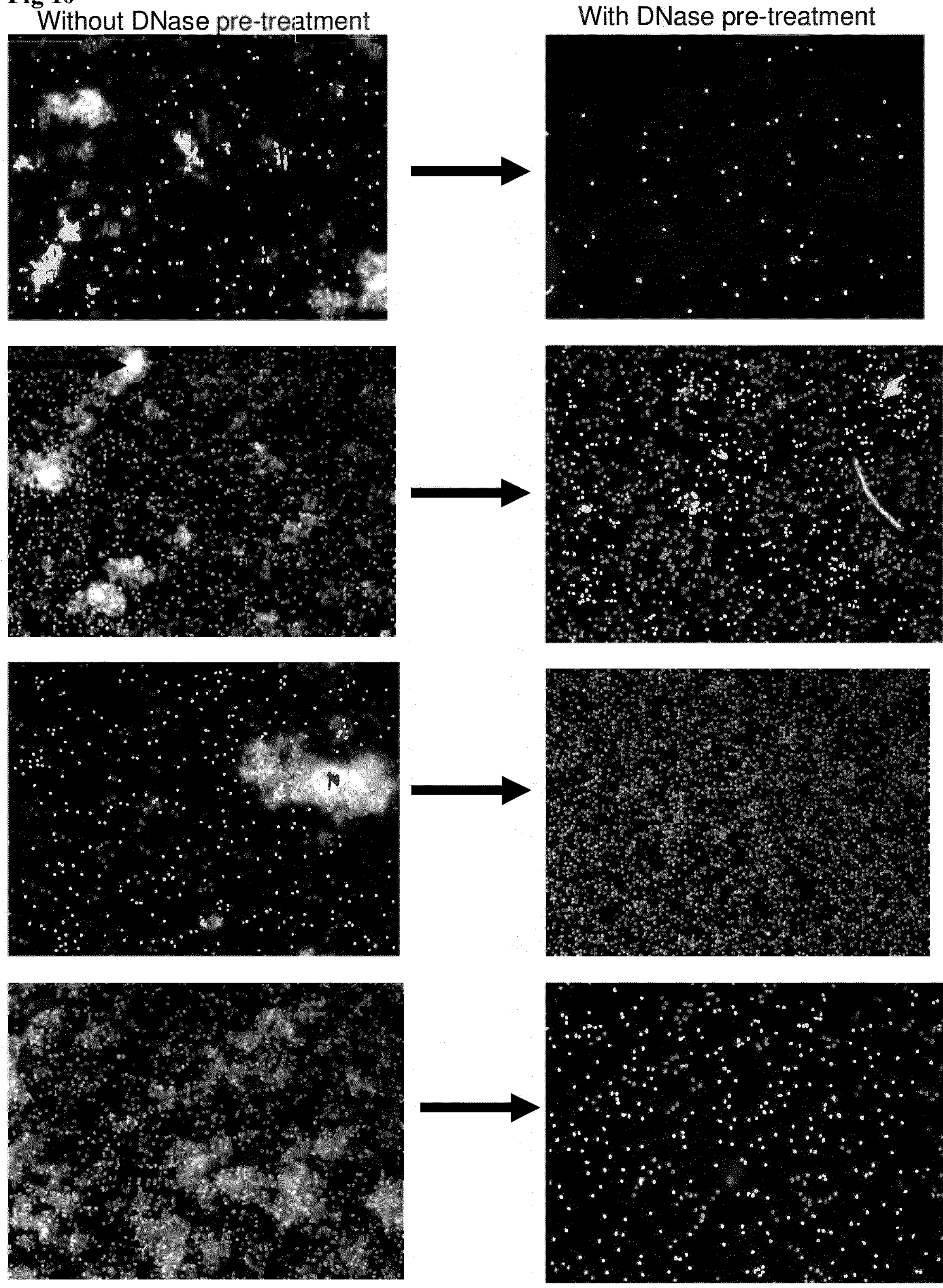


Fig 10



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/065704

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/574 G01N33/50 G01N33/53 G01N33/543
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LAMBROS MARYOU B. ET AL: "Single-Cell Analyses of Prostate Cancer Liquid Biopsies Acquired by Apheresis", CLINICAL CANCER RESEARCH , vol. 24, no. 22 15 November 2018 (2018-11-15), pages 5635-5644, XP093089263, US ISSN: 1078-0432, DOI: 10.1158/1078-0432.CCR-18-0862 Retrieved from the Internet: URL:https://aacrjournals.org/clincancerres/article-pdf/24/22/5635/2047630/5635.pdf cited in the application PG 5636,5641,5642 -----	1-14

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[x] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
18 July 2024	29/07/2024

Name and mailing address of the ISA/	Authorized officer
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1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TAMMINGA MENNO ET AL: "Leukapheresis increases circulating tumour cell yield in non-small cell lung cancer, counts related to tumour response and survival", BRITISH JOURNAL OF CANCER, NATURE PUBLISHING GROUP UK, LONDON, vol. 126, no. 3, 30 November 2021 (2021-11-30), pages 409-418, XP037682341, ISSN: 0007-0920, DOI: 10.1038/S41416-021-01634-0 [retrieved on 2021-11-30] PG 409,410, 414 -----	1-14
A	WU JUNHAO ET AL: "Magnetic-Based Enrichment of Rare Cells from High Concentrated Blood Samples", CANCERS, vol. 12, no. 4, 10 April 2020 (2020-04-10), page 933, XP055811083, DOI: 10.3390/cancers12040933 pages 2, 11 - page 13; figure 4; table 1 -----	1-14
A	RADFAR PAYAR ET AL: "Single-cell analysis of circulating tumour cells: enabling technologies and clinical applications", TRENDS IN BIOTECHNOLOGY, ELSEVIER PUBLICATIONS, CAMBRIDGE, GB, vol. 40, no. 9, 17 March 2022 (2022-03-17), pages 1041-1060, XP087145971, ISSN: 0167-7799, DOI: 10.1016/J.TIBTECH.2022.02.004 [retrieved on 2022-03-17] PG 1041,1045, Box 1; table 2 -----	1-14
A	STEVENS MICHIEL ET AL: "StarDist Image Segmentation Improves Circulating Tumor Cell Detection", CANCERS, vol. 14, no. 12, 13 June 2022 (2022-06-13), pages 1-13, XP093089293, DOI: 10.3390/cancers14122916 cited in the application PG 1,2,4,7,9 -----	1-14
X,P	MICHIEL STEVENS ET AL: "Improved enrichment of circulating tumor cells from diagnostic leukapheresis product", CYTOMETRY A, WILEY-LISS, HOBOKEN, USA, vol. 103, no. 11, 2 August 2023 (2023-08-02), pages 881-888, XP072529807, ISSN: 1552-4922, DOI: 10.1002/CYTO.A.24779 the whole document -----	1-14